Plant and Soil X, no. 4

April 1959

THE EFFECT OF MOLYBDENUM AND NITROGEN DEFICIENCIES ON NITRATE REDUCTION IN PLANT TISSUES *

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INTRODUCTION

The mechanism of nitrate reduction in fungi and higher plants has been cleared up by Nason and Evans [3 8] and Nicholas and Nason [11]. In experiments with purified cell-free extracts they found that the nitrate→nitrite reaction is catalyzed by an enzyme with either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as the prosthetic group and moreover molybdenum as a constituent. Reduced di- or triphosphopyridine nucleotide (DPNH or TPNH) are required as electron donor. The reduction sequence was established to be as follows:

$$\text{TPNH (or DPNH)} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

Recent investigations by Nicholas and Scawin [12] and by Kinsky and MacElroy [4] have shown that nitrate reductase requires inorganic phosphate for maximal activity. The latter authors furthermore found that their preparations of nitrate reductase isolated from Neurospora were able to reduce cytochrome c in the presence of TPNH.

In the reduction of nitrite to ammonia a number of enzymatic reactions are involved. Medina and Nicholas (see Nicholas [9]) have recently identified three metal-containing flavin enzymes in

* The major part of this work was carried out by the authors at the Institute for Soil Fertility, Groningen.

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cell-free extracts of Neurospora, viz. a nitrite reductase which reduces nitrite to hyponitrite, a hyponitrite reductase which reduces hyponitrite to hydroxylamine, and a hydroxylamine reductase which converts hydroxylamine to ammonia. There is an indication that the first two of these TPNH- or DPNH-requiring enzymes depend on the presence of copper or iron, and the third on manganese.

In the present study nitrate-reducing capacities of molybdenum-deficient cauliflower, spinach, and tomato plants were estimated at different periods of time after the addition of molybdate to the soil in which the plants were growing. In a number of experiments the molybdenum-deficient leaves were infiltrated with dilute solutions of sodium molybdate.

For comparison, the effect of the addition of nitrate or ammonium sulphate on the nitrate-reducing capacity of nitrogen-deficient cauliflower plants, well-supplied with molybdenum, was studied.

EXPERIMENTAL

Protein- and soluble non-protein-nitrogen were determined as described in a previous paper [7].

Nitrate was estimated according to the xylenol method [1].

Nitrate-reducing capacity of plant tissues was measured by using coarsely cut fragments of approximately 2 to 3 by 2 to 3 mm. One-gram portions were transferred to Thunberg tubes and provided with 5 ml 0.06 *M* phosphate buffer (pH 7.1), 1 ml 0.1 *M* malic acid neutralized with sodium hydroxide, 1 ml 0.1 *M* KNO_3 and 2 ml H_2O. After evacuation of the tubes air was slowly admitted to promote the uptake of the solution by the tissue fragments. Then the tubes were re-evacuated and incubated at 37°C for half an hour. The nitrite formed by the tissue fragments and released into the solution was determined according to the Griess-Romijn method [13] with a mixture of α-naphthylamine and sulphanilic acid.

Enzyme activity was stopped by adding 1 ml of acetic acid and the solution was cleared with 3 ml of a saturated solution of $(NH_4)_2SO_4$. Blank values, obtained by adding 1 ml of acetic acid and 3 ml of saturated $(NH_4)_2SO_4$ to the complete mixture before evacuation, were subtracted.

To study the effect of molybdenum and nitrate supply to cauliflower, spinach, and tomato plants on nitrate-reducing capacity of the tissues, the plants were grown as described in a previous paper [7] on molybdenum-deficient soil either in the absence of molybdenum with added nitrate, or in the presence of molybdenum without nitrate. The effect of molybdenum was studied by adding molybdate to the soil or by infiltrating it into the leaves of

molybdenum-deficient plants by vacuum infiltration. Similar treatments were used for nitrogen in the study of the effect of nitrate.

The infiltration of molybdate and nitrate solutions was performed by transferring leaves or leaf fragments to small beakers containing these solutions which were placed in an exsiccator. After evacuation, air was slowly admitted and as a result the pores of the leaf tissues were filled with liquid. The leaves were then exposed to an air stream to reduce their moisture content to the original level and incubated for different periods of time at room temperature in a moist atmosphere.

Malic-dehydrogenase activity in leaf tissue was estimated by transferring 0.5 g of tissue fragments to Thunberg tubes and adding 1 ml 0.1 *M* malic acid neutralized with sodium hydroxide, 0.5 mg DPN in 0.5 ml H_2O, 0.2 ml 0.2 *M* KCN, 0.5 ml 0.035% methylene blue, and 7.8 ml 0.06 *M* phosphate buffer (pH 7.0). The Thunberg tubes were evacuated before adding the methylene-blue solution, air was slowly admitted, methylene blue added, tubes again evacuated and incubated at 37°C. Time of reduction of methylene blue was taken as a measure of malic-dehydrogenase activity [5].

Catalase activity was estimated as follows. 0.2 to 0.5 g of ground plant material was transferred with 10 ml of 1/15 *M* phosphate buffer, pH 7.0, to a 1-l Erlenmeyer flask fitted with a small, calibrated separatory funnel containing a 6% solution of H_2O_2 in a similar phosphate buffer. The flask was further connected with a calibrated "Bunte" burette, filled with H_2O. Ten ml of H_2O_2 solution was added to the macerate while the flask was in connection with the atmosphere via the three-way stopcock of the burette.

Immediately after mixing the H_2O_2 and the plant extract, the stopcock was turned so that the amount of O_2 evolved by the catalase activity could be measured by the displacement of water from the burette. Care was taken that the readings were made at atmospheric pressure in the Erlenmeyer flask. During the test the flask was shaken constantly.

RESULTS

1. *Effect of molybdenum, added to molybdenum-deficient cauliflower, spinach, and tomato plants, on nitrate-reducing capacity of these plants*

a) Nitrate reduction in molybdenum-deficient plants upon addition of Na_2MoO_4 to the soil.

Cauliflower plants. Estimations of nitrate-reducing capacity were carried out in leaf fragments of molybdenum-deficient plants approximately $4\frac{1}{4}$, $9\frac{3}{4}$, and $24\frac{1}{4}$ hours after application of 2.5 mg molybdate to 1 kg of the molybdenum-deficient soil in which the plants were growing. As will be seen from Table I, A, a twentyfold increase of nitrate-reducing capacity was found after $4\frac{1}{4}$ hours. After an incubation

TABLE I

Effect of added molybdenum on nitrate-reducing capacity of molybdenum-deficient plants.*

Treatment of plants	Plant tissue	NO_3-reducing capacity **	Treatment of plants	Plant tissue	NO_3-reducing capacity **
A			0 (continued)		
Cauliflower			0 Mo, 1 day Mo	Stem	4.0
0 Mo, control	Leaf	0.3		Seed leaf	2.3
	Stem	0.4	0 Mo, 3 days Mo	Leaf	3.4
0 Mo, 4½ h Mo	Leaf	6.3		Stem	5.2
	Stem	2.0		Seed leaf	3.0
0 Mo, 9¾ h Mo	Leaf	7.0	0 Mo, 6 days Mo	Leaf	0.9
	Stem	2.7		Stem	2.8
0 Mo, 24½ h Mo	Leaf	4.7		Seed leaf	1.1
	Stem	2.7	+ Mo, control	Leaf	0.5
+ Mo, control	Leaf	3.1		Stem	1.2
	Stem	2.0		Seed leaf	0.7
B			**D**		
Cauliflower			*Tomato*		
0 Mo, ¼ h H_2O	Leaf	1.0	0 Mo, control	Leaf	0.2
0 Mo, 2 h Mo	Leaf	2.9		Stem	1.3
0 Mo, 2 h H_2O	Leaf	1.1	0 Mo, 1 day Mo	Leaf	2.1
0 Mo, 4¾ h Mo	Leaf	6.3		Stem	2.4
0 Mo, 4¾ h H_2O	Leaf	1.3	0 Mo, 2 days Mo	Leaf	1.2
				Stem	2.4
C			0 Mo, 5 days Mo	Leaf	1.2
Spinach				Stem	1.8
0 Mo, control	Leaf	0.0	0 Mo, 7 days Mo	Leaf	0.5
	Stem	0.5		Stem	2.4
	Seed leaf	0.5			
0 Mo, 1 day Mo	Leaf	3.7			

* Averages values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, in % of dry matter, respectively, of:

Cauliflower: **A**, 0 Mo control, leaf: 3.2, 1.7, 2.2; stem: 1.5, 1.1, 3.9; + Mo control, leaf: 4.4, 1.4, 1.1; stem: 1.5, 1.1, 3.3. **B**, 0 Mo, H_2O, leaf: 3.3, 1.8, 2.4.

Spinach: 0 Mo control, leaf: 2.8, 1.6, 3.1; stem: 1.9, 1.4, 3.4; seed leaf: 2.3, 1.1, 3.0; + Mo control, leaf: 4.5, 1.6, 0.7; stem: 2.3, 1.5, 2.7; seed leaf: 2.8, 1.4, 1.5; 3 and 6 days after treatment NO_3-N of the leaves had decreased to 2.7 and 1.8% respectively.

Tomato: 0 Mo control, leaf: 3.9, 0.9, 2.3; stem: 1.6, 0.6, 3.8; 2, 5 and 7 days after Mo treatment NO_3-N of the leaves had decreased to 1.9, 0.7, and 0.5% respectively.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per ½h per mg protein.

time of 9¾ h some further increase of nitrate-reducing power had occurred. After 24 hours considerably lower values were found in leaf fragments, but not in stem tissue.

In a subsequent experiment with cauliflower plants nitrate-

reductase activity was measured approximately two hours after the addition of molybdate to the soil in which the molybdenum-deficient plants were growing. In this case nitrate-reducing capacity of leaf fragments had increased to a threefold level of the control (Table I, B). Plants of the same series tested five hours after the addition of molybdenum, showed a much higher activity. Apparently under the conditions of these experiments (greenhouse, autumn, approximately 20°C) nitrate-reducing capacity in molybdenum-deficient cauliflower leaves reached its full activity 4 to 5 hours after the addition of molybdate to the soil in which the plants were growing.

Spinach plants. Estimations of nitrate-reducing capacity in leaf and stem fragments were carried out 1, 3, and 6 days after the application of molybdate. High values were observed 1 day after molybdenum treatment (Table I, C). Six days after the addition of molybdenum, the nitrate-reducing capacity of molybdenum-deficient plants had decreased to a level slightly higher than that of plants supplied with adequate amounts of molybdenum from the beginning of their development.

The molybdenum-deficient spinach plants were extremely rich in nitrate nitrogen; the highest value found, viz. 3.45%, corresponds with a nitrate content of approximately 25% of the dry matter. Three days after the addition of molybdenum, the nitrate contents of the leaves and seed leaves had clearly decreased whereas the protein values had increased.

Tomato plants. Two experiments with molybdenum-deficient tomato plants have been carried out. In the first one it was shown that $4\frac{1}{2}$ hours after the addition of molybdate to the soil, it had been taken up by the plants and had affected a pronounced rise in the nitrate-reducing capacity of the leaves.

In the second experiment, carried out in the second part of August 1954, nitrate-reductase activity was determined in leaves, stems and roots, 1, 2, 5, and 7 days after the addition of molybdenum. The highest value for leaf tissue was found 1 day after the application of molybdenum. After longer incubation times lower values were found (Table I, D). These results are in agreement with those of the preceding experiment with spinach plants. In stem tissue such variation did not occur.

b) Nitrate reduction in molybdenum-deficient plants upon infiltration with sodium molybdate.

Cauliflower plants. One half of molybdenum-deficient leaves was infiltrated with a solution containing 25 ppm of $Na_2MoO_4.2H_2O$, the other half with H_2O. It was shown that two hours after completion of the infiltration with molybdate, the nitrate reducing capacity had reached its maximal value (Table II, A).

Spinach plants. A comparison was made between nitrate-

TABLE II

Effect of infiltrated molybdate on nitrate reduction in Mo-deficient cauliflower, spinach, and tomato leaves.*

Treatment of plants and infiltrated solution	Incubation time, h	Nitrate-reducing capacity **	Treatment of plants and infiltrated solution	Incubation time, h	Nitrate-reducing capacity **
A			**B** (Spinach continued)		
Cauliflower			+ Mo, H_2O	1	3.1
0 Mo, H_2O	2	0.4	+ Mo, H_2O	4.25	4.1
0 Mo, Na_2MoO_4	2	5.8	+ Mo, Na_2MoO_4	1	3.3
0 Mo, Na_2MoO_4	4	4.2	+ Mo, Na_2MoO_4	4.25	4.5
B			**C**		
Spinach			*Tomato*		
0 Mo, no infiltration	0	0.6	0 Mo, H_2O	2	0.4
0 Mo, H_2O	1	2.2	0 Mo, Na_2MoO_4	2	2.4
0 Mo, H_2O	4.25	3.4	0 Mo, H_2O	4	0.4
0 Mo, Na_2MoO_4	1	4.3	0 Mo, Na_2MoO_4	4	4.9
0 Mo, Na_2MoO_4	4.25	8.4			
+ Mo, no infiltration	0	3.5			

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: *Cauliflower:* 2.9, 1.4, 2.3. *Spinach*, 0 Mo: 3.2, 1.5, 2.3; + Mo: 4.3, 1.5, 0.7. *Tomato:* 3.3, 1.2, 2.2.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per mg protein.

reducing capacities of molybdenum-deficient leaves infiltrated with a molybdate-containing solution (1 ml = 20 μg of $Na_2MoO_4.2H_2O$) and H_2O, respectively. Estimations of nitrate-reducing capacities were made 1 and $4\frac{1}{2}$ h after infiltration. The results of this experiment recorded in Table II, B, demonstrate that 1 hour after completion of the infiltration with sodium molybdate, nitrate-reducing capacity has been restored only partly. At $4\frac{1}{4}$ h, a value was obtained

which was considerably higher than that of normal plants, even when the latter had been infiltrated with molybdate.

Infiltration with H_2O alone increased nitrate reduction of molybdenum-deficient plants considerably. In the case of normal plants this H_2O-effect was also observed, but to a smaller extent.

Tomato plants. Coarsely cut leaf fragments of molybdenum-deficient plants were infiltrated with H_2O or a solution containing 20 ppm of $Na_2MoO_4.2H_2O$. Two and four hours, respectively, after completion of the infiltration, the leaf fragments were transferred to Thunberg tubes and nitrate-reducing capacities estimated. Two hours after the infiltration with molybdate, the molybdenum-deficient leaf fragments had not yet obtained their maximal nitrate-reducing capacity (see Table II, C).

2. *Effect of the nitrogen supply to cauliflower, spinach, and tomato plants on nitrate-reducing capacity*

Since nitrate reductase presumably is an adaptive enzyme, it was suggested that plants inadequately supplied with nitrate nitrogen would possess a lower nitrate-reducing capacity than those well supplied with this nutrient. Experiments were therefore done with nitrogen-deficient plants which had been supplied, either by application to the soil, or by infiltration, with nitrate or an ammonium compound.

a) Nitrate reduction in nitrogen-deficient plants upon addition of KNO_3 to the soil. Experiments were carried out with spinach and tomato plants. These plants were grown in similar Neubauer jars as used in experiments with molybdenum, while the same molybdenum-deficient soil was employed. This soil was also dressed in the same way except that $2\frac{1}{2}$ mg Na_2MoO_4 was given per 1 kg of soil, and nitrate was omitted.

In the case of spinach, the nitrogen-deficient plants were characterized by a much reduced development as compared with plants normally supplied with nitrate; their leaf colour was, however, only of a slightly lighter green. 0,75 g KNO_3 was added per pot on 5 October 1954 and harvests were made after 1, 2, 3, and 6 days. In addition to nitrate-reducing capacity, estimations were made of protein-N, soluble non-protein-N and nitrate-N. The results of these experiments are recorded in Table III, A, those of a similar experiment with tomato plants in Table III, B. It will be seen that ad-

TABLE III

Effect of added KNO_3 on nitrate-reducing capacity of nitrogen-deficient spinach and tomato plants.*

Treatment of plants	Plant tissue	NO_3-reducing capacity **	Treatment of plants	Plant tissue	NO_3-reducing capacity **
A			**B**		
Spinach			*Tomato*		
0 N, control	Leaf	0.8	0 N, control	Leaf	0.0
	Stem	2.1		Stem	3.1
	Seed leaf	0.1	0 N, 1 day KNO_3	Leaf	0.8
0 N, 2 days KNO_3	Leaf	4.3		Stem	7.6
	Stem	3.7	0 N, 2 days KNO_3	Leaf	0.4
	Seed leaf	2.5		Stem	4.1
0 N, 3 days KNO_3	Leaf	3.3	0 N, 5 days KNO_3	Leaf	1.9
	Stem	3.9		Stem	3.4
	Seed leaf	1.9	0 N, 7 days KNO_3	Leaf	1.1
0 N, 6 days KNO_3	Leaf	2.1		Stem	2.0
	Stem	2.8			
	Seed leaf	1.6			
+ N, control	Leaf	0.9			
	Stem	2.7			
	Seed leaf	0.5			

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter:

Spinach: 0 N control, leaf 3.7, 1.3, 0.1; stem 2.2, 1.7 0.0; seed leaf 1.8, 0.9, 0.1; 2 and 6 days after KNO_3 treatment NO_3-N in the leaves increased to 0.6 and 0.7% respectively.

Tomato: 0 N control, leaf 1.9, 0.3, 0.0; stem 0.7, 0.5, 0.0; 2 and 7 days after KNO_3 treatment NO_3-N in the leaves had increased to 0.4 and 0.7% respectively.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per mg of protein.

dition of nitrate to nitrogen-deficient plants brought about a pronounced rise of nitrate-reducing capacity. As with the molybdenum treatment of molybdenum-deficient plants this effect was most pronounced shortly after the application of the nitrate. After 6 or 7 days it was clearly reduced.

b) Effect of added nitrate and ammonium sulphate on nitrate-reducing capacity of nitrogen-deficient cauliflower leaves (28 October 1954). The nitrogen-deficient plants were considerably smaller than those dressed with nitrate, their leaf colour was of a slightly lighter green than normal, while the seed

leaves had died. Ten pots of this series were dressed with 5 ml of a solution containing 100 mg KNO_3 per ml. This solution was washed into the soil with 20 ml H_2O. A second series of ten pots was dressed with an equal amount of nitrogen in the form of $(NH_4)_2SO_4$, whereas a third series received H_2O only. At different periods of time after the addition of the nitrogenous compounds, plants were harvested and tested for nitrate-reducing capacity. The results of this experiment are recorded in Table IV. It will be seen that $4\frac{1}{2}$ h after the

TABLE IV

Effect of added KNO_3 and $(NH_4)_2SO_4$ on nitrate-reducing capacity of nitrogen-deficient cauliflower leaves *

Treatment of plants	Incubation time, hours	NO_3-reducing capacity **	Treatment of plants	Incubation time, hours	NO_3-reducing capacity **
H_2O, control	4.5	1.6	$(NH_4)_2SO_4$	23	2.7
KNO_3	4.5	4.3	H_2O, control	47	1.3
$(NH_4)_2SO_4$	4.5	3.2	KNO_3	47	6.4
H_2O, control	23	0.7	$(NH_4)_2SO_4$	47	3.4
KNO_3	23	5.1			

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: 2.0, 0.7 and 0.0.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per mg protein.

application of nitrate, the nitrate-reducing capacity of the leaves had considerably increased. After 1 and 2 days, respectively, this activity had still further increased. Plants dressed with ammonium sulphate also showed increased nitrate reduction though to a lesser extent than those treated with nitrate.

c) Infiltration of KNO_3 into nitrogen-deficient tomato leaves (1 November 1954). For this experiment plants with moderate symptoms of nitrogen deficiency were employed. Coarsely cut leaf fragments were infiltrated with a KNO_3-containing solution (1 ml = 10 mg) and H_2O, respectively. $2^2/_3$ and 5 h after infiltration, nitrate-reducing capacities were determined. As may be seen from the results in Table V, A, nitrate-reducing power had considerably increased $2^2/_3$ h after infiltration with a nitrate-containing solution. After 5 h a further increase had taken place.

d) Effect of infiltrated KNO_3, $(NH_4)_2SO_4$ and K_2SO_4 on nitrate-reducing capacity of nitrogen-deficient tomato plants. In order to investigate whether the nitrate-reducing capacity could be restored by infiltration of the leaves with solutions of KNO_3 or $(NH_4)_2SO_4$, an experiment was carried out with N-deficient tomato leaves on 5 November 1954. The infiltrations were done with solutions of KNO_3 (1 ml = 10 mg), $(NH_4)_2SO_4$ (1 ml = 6.5 mg) and K_2SO_4 (1 ml = 8.6 mg). The temperature during the incubation was 22°C. Five hours after the completion of the infiltration, the leaves were cut into coarse fragments and nitrate-reducing capacity was estimated. As will be seen from the data of Table V, B, nitrate-reducing capacity was restored only if the leaves had been infiltrated with KNO_3.

TABLE V

Effect of infiltrated KNO_3 and $(NH_4)_2SO_4$ on nitrate-reduction in nitrogen-deficient tomato leaves.

Treatment of plants and infiltrated solution	Incubation time, hours	Nitrate-reducing capacity **	Treatment of plants and infiltrated solution	Incubation time, hours	Nitrate-reducing capacity ***
A *			**B**		
0 N, H_2O	3	1.6	0 N, H_2O	$3\frac{1}{2}$	26
0 N, KNO_3	$2\frac{2}{3}$	2.9	0 N, KNO_3	$3\frac{1}{2}$	129
0 N, H_2O	5	1.0	0 N, $(NH_4)_2SO_4$	$3\frac{1}{2}$	16
0 N, KNO_3	5	4.1	0 N, K_2SO_4	$3\frac{1}{2}$	20

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: 3.2, 0.8, 0.0; after infiltration NO_3-N had increased to 0.6%.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per mg protein.

*** Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per g fresh weight.

That the effect of infiltrated KNO_3, in the preceding experiments, was due to the formation of an adaptive nitrate-reducing enzyme and not to an improved nitrate supply to an existing enzyme system, was concluded from an experiment using leaves of normal cauliflower plants grown in the presence of nitrate. These dark-green leaves contained no detectable nitrate and were assumed to have a normal enzyme content. Fragments of such leaves produced immediately – in an incubation period of half an hour – considerable quantities of nitrite when incubated in evacuated Thunberg systems in the presence of nitrate. Controls in the absence of added nitrate produced little nitrite and hence it must be concluded that this

substance originated by reduction of added nitrate. The absence of a lag phase in the development of reducing power suggests that nitrate reductase was initially present in these leaves grown on nitrate, whereas the delay in development of nitrate reduction in leaves grown in the absence of nitrate in previous experiments suggests adaptive enzyme formation.

e) Comparison of the effects of infiltrated KNO_3 on N-deficient and of infiltrated Na_2MoO_4 on molybdenum-deficient tomato plants. For this experiment tomato plants were grown in molybdenum-deficient soil a) with added molybdenum but without nitrate, b) with added KNO_3 but without molybdenum. The symptoms of nitrogen deficiency were less severe than those of molybdenum deficiency. The leaves of both series were harvested at 10 a.m. on 2 November 1954. The nitrogen-deficient samples were infiltrated with a solution of KNO_3 (1 ml = 10 mg); those from molybdenum-deficient plants were infiltrated with a solution containing 20 ppm $Na_2MoO_4.2H_2O$. Control samples of both series were infiltrated with H_2O only. Nitrate-reducing capacities of coarsely cut leaf fragments were estimated by the usual procedure 2, 4, and 6 hours after infiltration (Table VI).

TABLE VI

Nitrate-reducing capacities of nitrogen-deficient tomato leaves * infiltrated with KNO_3, and molybdenum-deficient tomato leaves * infiltrated with $Na_2MoO_4.2H_2O$

Solutions infiltrated in N-deficient leaves	Incubation time, hours	Nitrate-reducing capacity **	Solutions infiltrated in Mo-deficient leaves	Incubation time, hours	Nitrate-reducing capacity **
H_2O	2	0.9	H_2O	2	0.1
KNO_3	2	2.6	Na_2MoO_4	2	1.0
H_2O	4½	1.8	H_2O	4½	0.1
KNO_3	4½	6.3	Na_2MoO_4	4½	2.8
H_2O	6½	1.1	H_2O	6½	0.3
KNO_3	6½	5.8	Na_2MoO_4	6½	3.4

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: 0 N: 3.1, 0.9, 0.0; after infiltration NO_3-N had increased to 0.5%. 0 Mo: 3.1, 1.4, and 2.6%.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per ½ h per mg of protein.

From the results in Table VI it may be seen that two hours after completion of the infiltration the nitrate-reducing capacities of N-deficient plants had been restored to approximately 50 per cent and that of Mo-deficient plants to approximately 30 per cent.

3. *Other factors affecting nitrate-reducing capacity in leaf fragments*

a) Effect of cutting and grinding of leaves on nitrate reduction. To investigate whether the nitrite formation observed in leaf fragments in the preceding experiments must be attributed to intact or disruptured cells, experiments were carried out with leaf fragments of different sizes and with ground tissue. In the first experiment, spinach plants, grown in the presence of both nitrate and molybdate, were used. Leaves from these plants were cut into fragments of approximately 1 by 1, 2 by 2 and 5 by 5 mm, respectively, and tested for nitrate-reducing capacity as described above. The highest values were found in coarsely cut fragments, viz 52 μg $NaNO_2$ formed per 1 g fresh tissue per $\frac{1}{2}$ h at 37°C; fragments of 2 mm had formed 39 μg $NaNO_2$ under the same conditions and those of 1 mm only 15 μg. These results demonstrate that nitrate reduction in leaf fragments takes place mainly in undamaged cells. This was still more clearly shown when the leaf tissue was ground in a mortar. In the latter experiment a comparison was made between fragments cut to different sizes from normal cauliflower leaves and a macerate of the same amount of plant material. To investigate the possibility of nitrite binding or nitrite reduction, two samples of ground leaf tissue were provided with a certain amount of nitrite before evacuating the Thunberg tubes. The results of this experiment, recorded in Table VII, provide evidence that in addition to inactivation of the nitrate-reducing enzyme system, loss of nitrite occurs in leaf macerates. Nevertheless extracts of ground leaf tissue, infiltrated

TABLE VII

Effect of cutting and grinding of tomato leaves on nitrate-reducing capacity and nitrite removal.

Treatment of leaf tissue	Nitrate-reducing capacity *
1. Whole leaves	51
2. Fragments of 5 by 5 mm	65
3. Fragments of 1–2 by 1–2 mm	22
4. Leaf macerate	8
5. Leaf macerate + 70 μg $NaNO_2$	51
6. Washed residue of macerate	10
7. Fragments of 5 by 5 mm, infiltrated with leachings of treatment 6	54

* Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per $\frac{1}{2}$ g fresh tissue.

into leaf fragments, hardly affected their nitrate-reducing power. Hence the depressing effect of grinding on nitrate reduction is not due to the presence of inhibitory substances.

The disappearance of nitrite in ground leaf tissue was shown more clearly in an experiment with spinach. Five grams of leaf macerate were provided with 300 μg $NaNO_2$ in addition to malic acid and phosphate buffer in the usual concentration. The mixture was incubated in an evacuated Thunberg tube for $^1/_4$ h at 37°C and subsequently analysed for nitrite. Only 122 μg of $NaNO_2$ were found which indicates a loss of approximately 60 per cent of the added nitrite.

The effect of ground leaf tissue on nitrite apparently is partly chemical and partly enzymatic. This was concluded from an experiment in which a comparison was made between fresh and boiled samples of a macerate of cauliflower leaves. 0.7 g of this macerate supplied with 250 μg of $NaNO_2$ under the usual conditions brought about a loss of 139 μg of $NaNO_2$ in $\frac{1}{2}$ h at 37°C when used in the fresh state and of 103 μg when it had been boiled. It is also possible that the effect of boiling is due to loss of nitrite-binding by the protein.

b) Effect of added yeast extract on nitrate reduction in leaf macerate. If it is assumed that nitrate reduction in leaf tissue is brought about by the activity of nitrate reductase, it can be suggested that either desorganization of the enzyme system or lack of substrate (DPNH or TPNH) were responsible for the inactivation of the nitrate-reducing capacity in disruptured cells. Therefore a few experiments with yeast extract (which is a source of FAD) have been carried out.

For the preparation of yeast extract, 25 g of dried "Engedura"-yeast were rubbed in a mortar, dissolved in 100 ml boiling water, and boiled for a few seconds. After cooling, the solution was centrifuged and the clear yellow centrifugate used for the enzyme tests.

In the first two experiments with macerates of normal spinach leaves a remarkable effect of yeast extract on restoring nitrate reduction was observed (see Table VIII).

In the case of leaf fragments, yeast extract had no effect on nitrate reduction.

In subsequent experiments with spinach and cauliflower leaves the effect of added yeast extract was considerably less pronounced

TABLE VIII

Effect of yeast extract on nitrate-reducing capacity of ground spinach leaves

First experiment		Second experiment	
Treatment	NO_3-reducing capacity*	Treatment	NO_3-reducing capacity*
0.5 g leaf macerate	3	0.5 g coarsely cut leaf fragm.	61
0.5 g l.m. + 1 ml yeast extract, added before grinding	30	dito + 1 ml yeast extract	65
		0.5 g leaf macerate	4
0.5 g l.m. + 1 ml yeast extract added 20 min after grinding	19	dito + ¼ ml yeast extract	10
		dito + 1 ml yeast extract	33
		dito + 2 ml yeast extract	24
1 ml yeast extract	4	dito + 51 μg $NaNO_2$	22
0.5 g l.m. + 60 μg $NaNO_2$	29	1 ml yeast extract	3
		2 ml yeast extract	4

* Averages of duplicate values expressed as μg $NaNO_2$ formed per ½ hour ½ g fresh tissue at 37°C.

than in the above-mentioned two tests. This was presumably due to the fact that yeast extract itself caused a loss of added nitrite (2 ml yeast extract added to the usual reaction mixture in evacuated Thunberg tubes at 37°C reduced added nitrite in ½ h from 130 to 30 μg. This was not an enzymatic effect since it was also exerted by yeast extract which had been boiled twice.

When a correction was made for the nitrite-decomposing effect of both leaf macerate and yeast extract, a small but consistent nitrate reduction was observed in the leaf-macerate–yeast-extract mixtures of the later experiments.

c) Effect of illumination on nitrate-reducing capacity. A comparison was made between cauliflower plants kept in the dark or illuminated, for 3 hours, in a thermostate of approximately 20°C. No difference in nitrate-reducing capacity of leaf fragments was observed.

In a subsequent experiment, cauliflower plants, kept for two days in the dark at 20°C, showed a somewhat reduced nitrate-reducing capacity as compared with that of plants kept under greenhouse conditions (27 and 32 μg of $NaNO_2$ formed per 1 g of leaf fragments per ½ h, respectively). When the plants were kept first in the dark for one day and then exposed to light in the greenhouse for one day an increased nitrite production was found, viz 45 μg $NaNO_2$ per 1 g of leaf fragments.

4. *Effect of molybdenum and nitrogen supply of cauliflower, spinach, and tomato plants on malic-dehydrogenase activity*

If it is assumed that DPNH or TPNH is used as the substrate in nitrate reduction, then a dehydrogenase activity is required to keep these nucleotides in the reduced state. The importance of dehydrogenase activity for nitrate reduction in leaf fragments of tomato plants was demonstrated in an experiment in which malic acid was omitted from the usual reaction mixture. Thirty-three μg of $NaNO_2$ were formed per $\frac{1}{2}$ g of leaf fragments against 47 μg when 1 ml 0.1 *M* malic acid had been added and 43 μg in the presence of 1 ml 0.1 *M* succinic acid. The fact that both malic and succinic acid affected nitrite formation, indicates that both malic and succinic dehydrogenases were active.

Malic-dehydrogenase activity was estimated according to the above-mentioned method in a number of tissues which were also tested for nitrate-reducing capacity. Table IX gives the results of

TABLE IX

Effect of molybdenum supply to spinach and cauliflower plants on malic-dehydrogenase and nitrate-reductase activities

Spinach leaves			Cauliflower leaves		
Treatment	Malic dehydrogenase *	Nitrate-reductase **	Treatment	Malic dehydrogenase *	Nitrate-reductase **
0 Mo	35 (57)	9	0 Mo	61 (81)	15
0 Mo, 5 h after addition of Na_2MoO_4 + Mo	53 (75)	74	0 Mo, 2 h after addition of Na_2MoO_4	31 (>160)	42
	37 (53)	60	0 Mo	86 (>200)	17
			0 Mo, 4½ h Na_2MoO_4	53 (>140)	99
			0 Mo	70 (>120)	18

* Averages of duplicate values. Activity expressed as time (in minutes) required for complete reduction of added methylene blue by $\frac{1}{2}$ g leaf fragments. In parentheses, values obtained when KCN was omitted from the reaction mixture.

** Averages of duplicate values expressed as μg $NaNO_2$ formed per $\frac{1}{2}$ h per g fresh tissue.

two experiments in which the effect of molybdenum supply on malic-dehydrogenase activity was estimated. For comparison nitrate-reducing capacities are recorded. It will be seen that in the case of spinach leaves no difference in malic-dehydrogenase activity

was observed between molybdenum-deficient and normal leaf tissue, while added molybdate had a depressing effect on activity. In cauliflower leaves, however, added molybdenum clearly promoted malic-dehydrogenase activity.

In the case of nitrogen-deficient cauliflower, added nitrate which promoted nitrate-reducing capacity (cf Table IV) did not affect malic dehydrogenase. Addition of $(NH_4)_2SO_4$ which favoured nitrate reduction slightly, adversely affected malic dehydrogenase (Table X).

TABLE X

Effect of added KNO_3 and $(NH_4)_2SO_4$ on malic-dehydrogenase activity of cauliflower leaves.*

Treatment	Malic dehydrogenase **	Treatment	Malic dehydrogenase **
0 N	14 (>90)	—	
0 N, 4½ h KNO_3	13 (>90)	0 N, 4½ h $(NH_4)_2SO_4$	30 (>90)
0 N	12 (117)	—	
0 N, 23 h KNO_3	12 (105)	0 N, 23 h $(NH_4)_2SO_4$	21 (138)
0 N	11 (>100)	—	
0 N, 47 h KNO_3	14 (>100)	0 N, 47 h $(NH_4)_2SO_4$	29 (>100)

* Nitrate-reducing capacities in Table IV

** Time (in minutes) required for complete reduction of added methylene blue; in parentheses: values obtained without KCN. Averages of duplicate values.

5. *Effect of molybdenum supply on catalase activity of cauliflower, spinach, and tomato plants*

a) Comparison of molybdenum-deficient and normal cauliflower and spinach leaves. Readings were made after different periods of time (see Fig. 1). It will be seen that catalase activity of molybdenum-deficient plants was considerably less than that of normal plants. As a result of this effect, pronounced differences in behaviour of molybdenum-deficient and normal plants were sometimes obtained when these plants were placed in dilute solutions of H_2O_2. The former lost their turgescence within a short time whereas normal plants were not affected. These results were not consistent, however; in a number of cases, molybdenum-deficient spinach and tomato plants were not affected by H_2O_2.

b) Effect of added molybdate. In a few further experiments the effect of added molybdate on catalase activity of molybdenum-deficient spinach and tomato plants was investigated. Although normal leaf tissue had a considerably higher catalase activity than

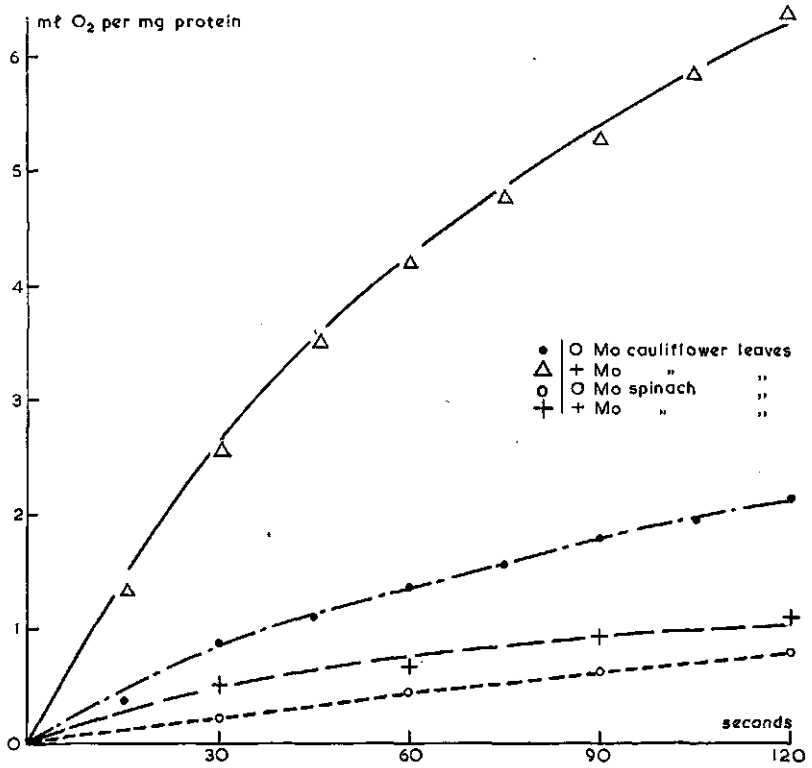

Fig. 1. The effect of molybdenum supply on the catalase activity (ml O_2 per mg protein) of cauliflower and spinach leaves. + Mo = 2.5 mg $Na_2MoO_4.2H_2O$ per pot. Averages of quadruplicate values.

molybdenum-deficient, the effect of added molybdate, at least during the first days after addition, was slight. In contrast to nitrate-reducing capacity of Mo-deficient plants which was always restored within one day after molybdenum treatment, catalase activity six days after molybdenum treatment had not yet reached the value of normal plants.

DISCUSSION

The effect of molybdenum on nitrate reduction in cauliflower, spinach, and tomato leaves was studied by adding molybdate to Mo-deficient plants or by infiltrating leaves of the latter plants with molybdate-containing solutions. Nitrate-reducing capacities of the

tissues were determined under anaerobic conditions at 37°C * at different periods of time after molybdate had been provided; malate served as hydrogen donor. Under these conditions relatively large amounts of nitrite were formed and released into the solution in which the tissue fragments were suspended. Since omission of malic acid from the reaction solution reduced nitrite formation, while added DPNH had no promoting effect, it was assumed that electron donors (DPNH or TPNH) are available or are produced in the tissue fragments in adequate amounts for optimal nitrate reduction.

Under the conditions of the experiments, it took approximately 4 hours to restore nitrate-reducing capacity to molybdenum-deficient leaf tissue by the addition of sodium molybdate. It was remarkable that in most cases the nitrate-reducing capacities of Mo-deficient tissues provided with molybdate were higher than those of normal tissues well supplied with molybdenum from the beginning of their development. This indicates the accumulation of some stimulating compound in molybdenum-deficient plants.

The experiments with nitrogen-deficient cauliflower, spinach, and tomato plants provided evidence for the fact that nitrate reductase in these plants is an adaptive enzyme. The beneficial effect of ammonium sulphate on the nitrate reduction of nitrogen-deficient cauliflower plants, observed $4\frac{1}{2}$ hours after its addition to the soil (Table IV) may have been due either to a very rapid nitrification of part of the added $(NH_4)_2SO_4$ or to the presence of some nitrate impurities in the ammonium-sulphate solution. That $(NH_4)_2SO_4$ itself has no effect on the nitrate-reducing capacity was concluded from the infiltration experiment recorded in Table V. These results are in agreement with those of Candela *et al.* [2] who found in cauliflower plants grown with ammonium sulphate as the nitrogen source, values for nitrate-reductase activity one-third and one-sixth of those observed in plants given nitrate or nitrite. Nicholas and Nason [11] obtained similar results with fungi.

The fact that a few hours after the addition of nitrate to the soil, the nitrate-reducing capacity of the leaves had increased to a large extent, makes it highly improbable that the restoration of the enzyme activity was due to the synthesis of entirely new protein from assimilated nitrate.

* At 20, 28 and 45°C lower values were obtained.

The experiments with leaf fragments of different sizes and with leaf macerates (Table VII) have provided evidence that the nitrate-reductase activity of the leaf fragments depends on the presence of undamaged cells. Grinding of the cells apparently inactivates the enzyme system; in addition it may disorganize the electron-donor system. Furthermore a loss of added nitrite was observed in leaf macerates. The fact that added yeast extract, which is a source of FAD, in some cases restored to a great extent the nitrate reduction of ground leaf tissue, but in other cases restored it only partly or not at all, may indicate that varying concentrations of reduced nucleotides were, at least partly, responsible for the irregular results of ground leaf tissues in the presence of yeast extract. That added FAD and TPNH are able to restore nitrate-reductase activity of ground leaf tissues may be concluded from the experiments of Evans and Nason [3] and Candela *et al.*[2].

Illumination of cauliflower plants was found to have a depressing effect on nitrate-reducing capacity only if the plants were previously kept in the dark for a prolonged period of time. This is in agreement with the results of similar experiments of Candela *et al.*[2].

The effect of molybdenum on the nitrate-reducing capacity of leaf fragments as observed in the present investigation may have been due, in addition to its effect on nitrate reductase, to some extent to the influence on malic-dehydrogenase activity, *i.e.* on providing reduced nucleotides. This is concluded from the fact that malic-dehydrogenase activity in some experiments was clearly promoted by added molybdate. That the reduction of methylene blue, which was used as a measure for malic-dehydrogenase activity, was really brought about by the activity of this enzyme was concluded from the fact that omission of KCN from the reaction medium retarded methylene-blue decolourization to a large extent.

The effect of molybdenum on malic dehydrogenase was less consistent and far less pronounced than that on nitrate-reducing capacity, however.

A further effect of molybdenum, which had been noticed earlier [6 10] is its influence on catalase activity. This is an indirect effect, however, and mainly depends, it is presumed, on newly synthesized protein. This is concluded from the slow rise in catalase activity upon addition of molybdate to molybdenum-deficient plants.

SUMMARY

The effect of molybdenum on the nitrate-reducing capacity of plant tissue was studied by using coarsely cut tissue fragments from plants grown in Mo-deficient soil and dressed or infiltrated with a molybdate-containing solution shortly before testing. The tissue fragments were suspended under anaerobic conditions in a nitrate-containing buffer solution with malate serving as a hydrogen donor. Formation of nitrite was used as a measure for the nitrate-reducing power. Very low values for nitrate-reducing capacity were found in molybdenum-deficient tissues. As little as four hours after the application of molybdenum, however, enzyme activity had reached its maximal value.

Cauliflower, spinach, and tomato plants, grown with inadequate amounts of nitrogen, showed also very low nitrate-reducing activities. Addition of nitrate to these plants, either by application to the soil or by infiltrating it into the leaves, resulted in a rapid rise in nitrate-reducing capacity. Ammonium sulphate, added to the soil, had some beneficial effect on nitrate reduction in the leaves of cauliflower plants but in the case of infiltration no effect was observed.

Evidence was obtained that the nitrate-reducing activity of leaf fragments depends on the presence of undamaged cells. Grinding of the cells inactivates the enzyme system; in addition it may desorganize the electron-donor system. Furthermore a loss of nitrite may occur in leaf macerates. Addition of yeast extract, which is a source of flavin adenine dinucleotide, a co-enzyme of nitrate reductase, restored in some cases the nitrate-reductase activities of leaf macerates to a large extent. In other cases its effect was slight or negligible.

Plants kept in the dark for a prolonged period of time showed lower nitrate-reducing capacities than those kept under normal light conditions.

In some experiments the malic-dehydrogenase activity of molybdenum-deficient plant tissues was found to be promoted by the addition of molybdate. This effect was less consistent and far less pronounced than that on nitrate-reducing capacity.

Molybdenum-deficient plant tissues usually have a lower catalase activity than plants supplied adequately with molybdenum.

Received July 9, 1958

ACKNOWLEDGEMENT

The authors are much indebted to Dr. J. L. Harley, Department of Botany, University of Oxford, for reading and improving the text of this paper.

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